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Evaluation of dispersive liquid–liquid microextraction for the simultaneous determination of chlorophenols and haloanisoles in wines and cork stoppers using gas chromatography–mass spectrometry

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ABSTRACT

Dispersive liquid–liquid microextraction (DLLME) coupled with gas chromatography–mass spectrometry (GC–MS) was evaluated for the simultaneous determination of five chlorophenols and seven haloanisoles in wines and cork stoppers. Parameters, such as the nature and volume of the extracting and disperser solvents, extraction time, salt addition, centrifugation time and sample volume or mass, affecting the DLLME were carefully optimized to extract and preconcentrate chlorophenols, in the form of their acetylated derivatives, and haloanisoles. In this extraction method, 1 mL of acetone (disperser solvent) containing 30 μL of carbon tetrachloride (extraction solvent) was rapidly injected by a syringe into 5 mL of sample solution containing 200 μL of acetic anhydride (derivatizing reagent) and 0.5 mL of phosphate buffer solution, thereby forming a cloudy solution. After extraction, phase separation was performed by centrifugation, and a volume of 4 μL of the sedimented phase was analyzed by GC–MS. The wine samples were directly used for the DLLME extraction (red wines required a 1:1 dilution with water). For cork samples, the target analytes were first extracted with pentane, the solvent was evaporated and the residue reconstituted with acetone before DLLME. The use of an internal standard (2,4-dibromoanisole) notably improved the repeatability of the procedure. Under the optimized conditions, detection limits ranged from 0.004 to 0.108 ng mL^{-1} in wine samples (24–220 pg g^{-1} in corks), depending on the compound and the sample analyzed. The enrichment factors for haloanisoles were in the 380–700-fold range.

1. Introduction

The wine industry considers aroma to be of great importance to product quality and consumer acceptance. The appearance of a defect in wines detected as a corked, musty, mouldy or with earthy off-flavour is normally related to the presence of some chlorophenols and chloroanisoles [1]. The main compound responsible for this defect is 2,4,6-trichloroanisole (2,4,6-TCA), although other chemicals, such as 2,3,4,6-tetrachloroanisole (2,3,4,6-TeCA), pentachloroanisole (PCA), 2,4-dichloroanisole (2,4-DCA) and 2,6-dichloroanisole (2,6-DCA), may also contribute to the off-flavours. These compounds are synthetized by fungal methylation of the corresponding chlorophenols [2]. 2,4,6-Tribromoanisole (2,4,6-TBA) has also been identified as a compound related with corked wines [3]. Although the term "corked" refers the defect to the natural cork used as bottles stopper, other sources may be responsible for spoilage in wines and attribution of the defect exclusively to cork stoppers is erroneous [1]. Chlorophenol compounds may also reach wine samples as a consequence of their use as biocides on wooden pallets and packing materials, their employment during production of bark cork and the further elaboration of cork stoppers or by the use of hypochlorite solutions in the cleaning of wooden barrels [4].

Gas chromatography is generally used for determining chlorophenols [4–7] and haloanisoles [3,4,8–26] in wines and, similarly, chlorophenols [27] and haloanisoles [2,9,10,15,28–35] in cork stoppers. The literature contains a relatively low number of references dealing with the simultaneous determination of chlorophenols and haloanisoles in wines [4,36,37] and in corks [38–41]. The volatility and thermostability of the latter make them suitable analytes for GC, whereas, in the case of chlorophenols, a previous derivatization step is always recommended before GC to improve sensitivity and reduce peak tailing.

A variety of preconcentration techniques have been applied for halophenols and haloanisoles in wine samples: classical techniques, such as liquid–liquid extraction (LLE) [3,10] and solid-phase extraction (SPE) [4,23,38,42], and the so-called clean chemistry techniques, such as purge-and-trap (PT) [15], stir bar sorptive extraction (SBSE) [13,17], pervaporation (PV) [19–21,24] and, to an even greater extent, solid-phase microextraction (SPME) [2,5–9,11,12,16,18,22,24–26,36,37]. Most of the procedures pro-

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posed for cork stoppers involve a previous solid-liquid extraction step [10,15,27,30,31,38–41] or supercritical fluid extraction (SFE) [29], although, if preconcentration is carried out by SPME in the headspace mode [2,9,32–35], this step can be avoided. Dispersive liquid–liquid microextraction (DLLME) is a very simple and rapid extraction, based on the use of a ternary component solvent system, which has been applied for the extraction and preconcentration of both organic and inorganic compounds from aqueous samples [43,44]. The low consumption of time and organic solvents are two of the main advantages of this technique, which may be included in the group of clean chemistry procedures. For its part, DLLME has been used for chlorophenols analysis in water samples [45,46] and other compounds in wine matrices [47,48]. Nevertheless, as far as we know, this preconcentration technique has not been used for the simultaneous analysis of chlorophenols and haloanisoles in wines and cork stoppers, as is the case of the procedure here proposed.

2. Experimental

2.1. Chemicals

4-Chloroanisole (4-CA, 99%), 2,4-dichloroanisole (2,4-DCA, 97%), 2,6-dichloroanisole (2,6-DCA, 97%), 2,4-dibromoanisole (2,4-DBA, 98%), 2,4,6-trichlorophenol (2,4,6-TCP, 99%), 2,4,6-tribromoanisole (2,4,6-TBA, 99%) and pentachlorophenol (PCP, 98%) were purchased from Aldrich (Steinheim, Germany). 4-Chlorophenol (4-CP, 99.5%), 2,6-dichlorophenol (2,6-DCP, 99.5%), 2,3,4,6-tetrachlorophenol (2,3,4,6-TeCP, 98%), 2,4,6-trichloroanisole (2,4,6-TCA, 99.5%) and 2,3,4,5-tetrachloroanisole (2,3,4,5-TeCA, 99%) were obtained from Dr. Ehrenstorfer (Ausburg, Germany) and pentachloroanisole (PCA, 99.3%) from Supelco (Bellefonte, PA, USA). Individual stock solutions of the compounds (1000 $\mu g\,mL^{-1}$) were prepared using HPLC grade methanol as a solvent and stored in darkness at −10 °C. Working standard solutions were freshly prepared in pure water and stored at 4 °C. Acetic anhydride, ethanol, anhydrous potassium carbonate, sodium dihydrogen phosphate and phosphoric acid (85%) were purchased from Fluka (Buchs, Switzerland) and sodium chloride of 99.5% purity from Sigma (St. Louis, MO, USA). Tartaric acid (99.5%) was purchased from Merck (NJ, USA). Chromatographic quality carbon tetrachloride, 1,2-dichlorobenzene, dichloromethane, chloroform, pentane, acetone, acetonitrile and methanol were obtained from Sigma. Water used was previously purified in a Milli-Q system (Millipore, Bedford, MA, USA). The carrier gas used for GC was helium (Air Liquide, Madrid, Spain).

To study the possibility of overcoming matrix effects, a synthetic wine containing 3.2 $g\,L^{-1}$ of L-(+)-tartaric acid and 12% (v/v) of ethanol, with the pH adjusted to 3.2 using a diluted NaOH solution was also prepared.

2.2. Instrumentation

GC analyses were performed on an Agilent 6890N (Agilent, Waldbronn, Germany) gas chromatograph coupled to an Agilent 5973 quadrupole mass selective spectrometer equipped with an inert ion source and provided with a split-splitless injection port. The helium carrier gas was maintained at a constant flow of 1 $mL\,min^{-1}$. A Zebron ZB-5ms (5% phenylarylene 95% dimethylpolysiloxane, Phenomenex, USA) capillary column (30 m × 0.25 mm i.d., 0.25 μm film thickness) was used. A ZB-1ms (100% dimethylpolyxilosane) capillary column (30 m × 0.25 mm i.d., 0.25 μm film thickness) was also tested. Injection volumes of 4 μL were used. The injection port was held at 250 °C and used in the splitless mode, applying a pressure pulse of 60 psi. The GC temperature was programmed as follows: start temperature of 50 °C (held 1 min) and increase to 115 °C at 15 °C min^{-1} and then to 160 °C at 3 °C min^{-1} (held 2.5 min). The total analysis time for one GC run was 23 min. The ionization was carried out in the electron-impact (EI) mode (70 eV). The electron multiplier voltage was set automatically. The temperatures of the ion source and the transfer line were 230 and 325 °C, respectively. The identification of the compounds was confirmed by injection of pure standards and comparison of their retention index and relevant MS-spectra. The analytes were quantified under the selected ion monitoring (SIM) mode using the target ion. Identification was confirmed by the retention time of the target ion and the qualifier-to-target ion ratios for acetylchlorophenols and haloanisoles (Table 1).

An IKA A11 grinder (IKA, Staufen, Germany) and an ultrasonic probe processor UP 200H (Dr. Hielscher, Germany) were used for treating the cork samples. A Büchi vacuum V-500 rotatory evaporator R-200 coupled to a Büchi heating bath B-490 (Switzerland) was used to concentrate the sample extracts. An EBA 20 (Hettich, Tuttlingen, Germany) centrifuge was used at the maximum speed supported by the conical glass tubes, 5000 rpm. A laboratory-made system built in the Central Laboratory Service of the University of Murcia and consisting of a drilled block equipped with an electronic temperature control system was used to heat the corks in the presence of the simulated extraction solution.

2.3. Sample preparation

A total of 10 wines (5 red wines and 5 white wines) were obtained from a local supermarket. The corresponding cork stoppers were separately ground, weighed and placed in 50 mL polyethylene closed flasks before storing at −20 °C. In addition, unused corks were obtained from a local dealer. The cork samples were natural and agglomerates, these latest having been shown to release more of their endogenous haloanisoles into the wines than natural corks [38]. A tainted red wine sample was obtained from a local cellar. Samples were kept at 4 °C until analysis, in order to prevent losses of the most volatile analytes.

The white wine samples (5 mL) were directly submitted to extraction, while red wines were diluted with water in a 1:1 proportion (2.5 mL red wine and 2.5 mL water).

For the analysis of cork samples, a previously described extraction step [15] was applied. Forty milliliters of pentane were added to the ground cork and the mixture was sonicated for 2 min (60% amplitude) by means of a probe directly immersed into the solution. The supernatant was filtered through filter paper and the residue submitted to a second extraction step by adding 40 mL of pentane and sonicating for 1 min. The combined filtered extracts were concentrated to dryness using a rotatory vacuum evaporator at 25 °C. The residue obtained was reconstituted in 2 mL of acetone containing 30 μL CCl_4 and this extract was submitted to DLLME.

2.4. DLLME procedure

For DLLME, a 5 mL aliquot of the wine sample was placed in a 10-mL screw cap glass tube with conical bottom and spiked with 50 μL of a 5 $\mu g\,mL^{-1}$ solution of 2,4-DBA (as internal standard). Volumes of 0.5 mL of a phosphate buffer solution (0.7 M, pH 11) and 200 μL of acetic anhydride were added and the mixture was gently shaken. Then, 1 mL of acetone (dispersive solvent) containing 30 μL of CCl_4 (extraction solvent) was rapidly injected into the sample solution using a syringe, and the mixture was again gently shaken manually for several seconds. A cloudy solution consisting of very fine droplets of CCl_4 dispersed into the sample solution was formed, and the analytes (haloanisoles and acetylated chlorophenols) were extracted into the fine droplets. After centrifugation for 3 min at 5000 rpm, the extraction solvent was sedimented at the bottom of

Table 1
Retention time and target and qualifier ions for the analytes.

Name	Retention time, min	T	Q_1 (Q_1/T %)	Q_2 (Q_2/T %)	Q_3 (Q_3/T %)
4-CA	6.30	142	127 (57)	144 (29)	
2,6-DCA	7.28	176	161 (90)	133 (72)	178 (64)
4-CP	7.56	128	130 (28)	170 (9)	
2,4-DCA	8.56	176	161 (90)	133 (72)	178 (64)
2,4,6-TCA	9.02	195	210 (69)	212 (62)	167 (52)
2,6- DCP	9.15	162	204 (13)		
2,4,6-TCP	11.13	198	196 (96)	240 (7)	238 (6)
2,4-DBA	12.18	266	251 (58)	264 (48)	268 (39)
2,4,6-TBA	15.69	346	344 (94)	329 (72)	331 (69)
2,3,4,6-TeCP	15.87	232	230 (88)	272 (7)	
2,3,4,5-TeCA	17.01	246	201 (62)	231 (54)	
PCA	18.21	265	280 (98)		
PCP	20.89	268	264 (98)	306 (18)	310 (11)

the conical tube (the volume was about 10 μL). Four microlitres of the sedimented phase were removed with a 5 μL microsyringe and injected into the GC–MS.

For the cork samples, a 5 mL aliquot of water was placed in a 10-mL screw cap glass tube with conical bottom and spiked with the internal standard. Volumes of 0.5 mL of a phosphate buffer solution (0.7 M, pH 11) and 200 μL of acetic anhydride were added and the extract, reconstituted with 2 mL of acetone containing 30 μL of CCl_4, was injected. The rest of the DLLME procedure was carried out as indicated above.

2.5. Migration studies using food simulants

In order to estimate the release of the target analytes from cork stoppers into wines, migration studies were carried out using cork macerates obtained in accordance with Directive 93/8/EEC, which establishes mandatory guidelines for verifying the migration of components of material and plastic objects that come into contact with food products. The food simulants are classified by convention as having the character of one or more food types. Thus, tartaric acid was also added to the simulant containing 15% ethanol in order to achieve similar conditions to wine [49]. Ground cork (2.5 g, which roughly corresponds to the mass of one cork stopper), fortified at a concentration level of about 0.2 $\mu g\,g^{-1}$, was soaked in 25 mL of an aqueous solution containing 15% (v/v) ethanol and 5 $g\,L^{-1}$ tartaric acid for 10 days, with the mixture thermostated at 40 °C. Three replicates were analyzed at each fortification level. The mixtures were maintained in 40 mL amber glass vials sealed with hole-caps and PTFE/silicone in order to prevent analyte evaporation.

2.6. Recovery assays

Since no reference materials were available, spiked samples were prepared. Wine samples were spiked with concentrations ranging roughly between 5- and 10-fold the corresponding quantification limit of each analyte. The fortification procedure was applied to two different red wines and two different white wines and two replicates were analyzed in each case.

For the recovery study in cork samples, 10 unused agglomerated cork stoppers, preliminary submitted to the entire extraction and analysis procedure in order to verify the absence of the analytes, were used as blank matrix. The analytes were added in the 0.4–8 $ng\,g^{-1}$ concentration range, depending on the compound, roughly corresponding to 5- and 10-fold the quantification limits. Samples were vigorously shaken to homogenize the mixture and set aside for 60 min at room temperature to allow the solvent to evaporate before being submitted to the described above extraction procedure.

3. Results and discussion

3.1. Chromatographic parameters

The optimal separation conditions for the haloanisole and acetylated chlorophenol compounds were compared using two different stationary phases: ZB-1ms and ZB-5ms capillary columns. No significant differences were observed between the stationary phases but, because analyte retention times were about 1 min lower with the phase containing a 5% phenylarylene in the dimethylpolysiloxane structure, this capillary column was selected. All compounds eluted when the oven temperature was slowly increased from 115 to 160 °C, except the PCP derivative which eluted only when the column was maintained at 160 °C. Separation was carried out at a constant flow-rate of 1 $mL\,min^{-1}$. The effect of the injection temperature was studied between 200 and 300 °C. Higher analytical signals were attained for all the compounds at 250 °C, and this value was adopted. Higher sensitivity was attained for all the analytes when a 4 μL volume was injected in the splitless mode. When injecting high volumes in the splitless mode, the application of a pressure pulse during the injection can improve sensitivity and repeatability because the sample is introduced more rapidly into the column than when no pulse is applied. Therefore, pressure pulses of 20, 40 and 60 psi were assayed, the highest increase in sensitivity being attained with 60 psi.

3.2. Derivatization reaction

Aqueous acetylation is one of the most efficient, simple and fast derivatization reactions for chlorophenols, and consequently acetic anhydride was used. A basic medium is needed for the derivatization reaction to proceed, and so a study of the effect of pH on the chlorophenols extraction efficiency was carried out by adding potassium carbonate to the aqueous solutions. Best sensitivity was attained at pH 11 (obtained by 0.75% (w/v) K_2CO_3) and this condition could be used for the DLLME optimization. Nevertheless, when the preconcentration step was applied to wine samples using potassium carbonate to increase the pH, small bubbles were observed in the settled phase which worsened repeatability for the phase recovering. Taking into account that a basic pH is needed not only for the derivatization reaction but also for to avoid the extraction of cinnamic acid and its possible derivatives present in wine samples [50], phosphate buffer solutions were assayed at pH values ranging from 8 to 12. In this way, the formation of bubbles was prevented and no repeatability problems were observed when the settled phase was sampled. A pH value of 11 was attained by adding 0.5 mL of a 0.7 M phosphate buffer solution. On the other hand, when different volumes, ranging from 50 to 500 μL, of acetic anhydride were added to a fortified wine sample, a volume of 200 μL of the derivatization reagent provided the best sensitivity. Different

derivatization times, ranging from 30 s to 5 min, were assayed and no differences in sensitivity were observed. A mixture shaking time of one minute was finally adopted.

3.3. DLLME parameters

The parameters, such as the type and volume of both extraction and disperser solvents, the salt addition and the centrifugation time, affecting the DLLME procedure were optimized. For this purpose, 5 mL of an aqueous solution containing analyte concentrations of about 50 ng mL^{-1} was used and 4 μL of the settled phase was injected into the GC.

3.3.1. Experimental parameters

The correct choice of selection of extraction solvent is crucial for optimizing the DLLME procedure: it should show low solubility in water, high affinity for the analytes and good chromatographic behaviour, while its density should be greater than that of water. Carbon tetrachloride, 1,2-dichlorobenzene, dichloromethane and trichloro-methane were tried for the purpose by using 30 μL in 1 mL acetone as the disperser solvent and carbon tetrachloride was finally selected.

Taking into consideration the recommended order of DLLME parameter selection for good performance [44], the disperser solvent was selected next. Acetone, methanol and acetonitrile are miscible in the extraction solvent and in the aqueous solution and so were assayed by rapidly injecting 1 mL of each disperser containing 30 μL of CCl_4 into 5 mL of the aqueous solution. The extraction efficiency was higher when using acetone (Fig. 1A), with the additional advantage of its low toxicity [51].

The influence of the carbon tetrachloride volume was studied in the 10–40 μL range. As shown in Fig. 1B, the highest sensitivity was achieved when 30 μL CCl_4 was used, and this volume was selected. The volumes assayed for the disperser solvent were 0.25, 0.5, 1 and 2 mL, containing in all cases the extraction solvent volume at the previously optimized value. The organic mixture was injected into 5 mL of the aqueous solution containing the analytes. No significant differences were noted when using 1 or 2 mL of the disperser solvent (Fig. 1C) and so, finally, 1 mL acetone was selected.

Extraction time in DLLME is defined as the interval time between injecting the mixture of disperser and extraction solvents and before starting the centrifugation step. As expected, no differences in sensitivity were attained in the interval 30 s to 5 min, demonstrating that DLLME is practically time-independent, one of its most important advantages. Therefore, the mixture was shaken for a few seconds and then centrifuged, the centrifugation step being the most time-consuming. The effect of centrifugation time was studied for 1, 3 and 5 min at 5000 rpm (Fig. 2A) and 3 min was finally adopted.

To study the ionic strength effect, the experiments were carried out at different sodium chloride concentrations in the aqueous solution, ranging from 0 to 10% (w/v). The results showed that the extraction efficiency decreased for both haloanisoles and acetyl-chlorophenol compounds when the salt concentration was increased (Fig. 2B). Therefore, the addition of NaCl to the extraction solution was discarded.

3.3.2. Wine sample volume and cork mass

For white wines, 5 mL of the sample were used, but the complex matrix of red wines makes it necessary to dilute the sample prior to DLLME.

In the case of cork stoppers, the mass corresponding to one cork stopper (about 2.5 g for natural corks and up to 3 g for agglomerates) was submitted to the above-described procedure. The extraction step from the cork stoppers was carried out by applying a previously optimized procedure based on a double extraction in pentane assisted by ultrasounds by means of a probe, which allows total analyte extraction in the organic solvent in 3 min. Preliminary experiments were carried out in order to reconstitute the extract in 5 mL of water, before submission to the optimized DLLME procedure. Nevertheless, a solid residue was observed in the bottom of the flask once the pentane extract has been evaporated at vacuum. This was not dissolved with water or 15% (v/v) ethanol even when the mixture was immersed in an ultrasonic bath for 5 min. Recovery values obtained when the aqueous or ethanolic phases were submitted to derivatization and DLLME were lower than 60% in all cases. The best results were obtained when the residue was reconstituted in 2 mL of acetone containing 30 μL CCl_4 and this extract was submitted to DLLME by mixing with 5 mL of water to which the phosphate buffer solution, the acetic anhydride and the internal standard were added.

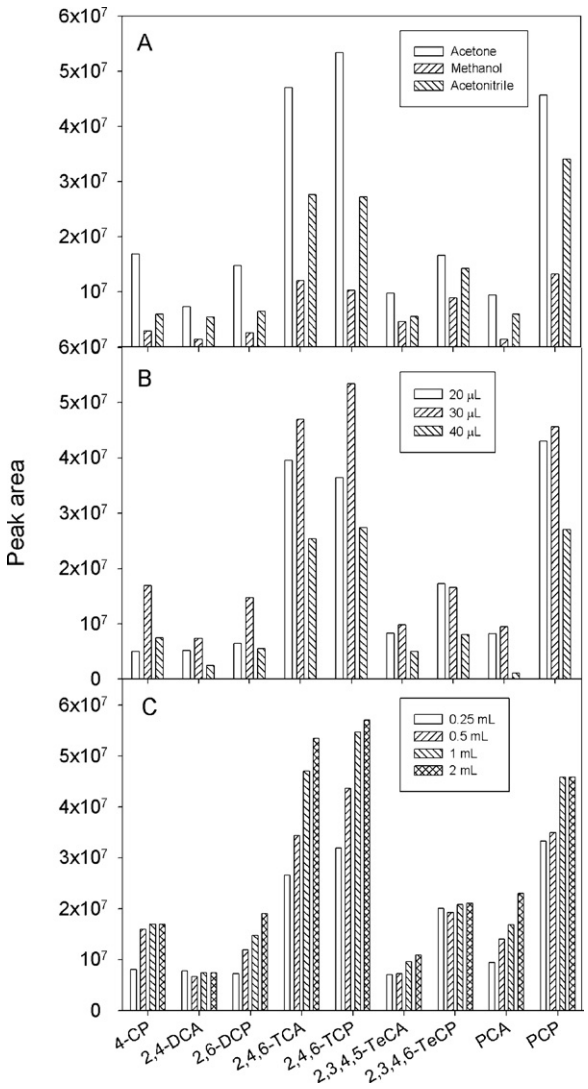

Fig. 1. Effect of (A) different disperser solvent, (B) the volume of carbon tetrachloride and (C) the volume of acetone on the sensitivity of various analytes by DLLME. Extraction conditions: aqueous phase, 5 mL; concentration of each compound, 50 ng mL^{-1}.

3.4. Analytical characteristics of the methods

3.4.1. Wine samples

The matrix effect was studied by comparing the slopes of aqueous standards and standard additions calibration graphs for six samples of different wines, obtained by plotting concentration (at

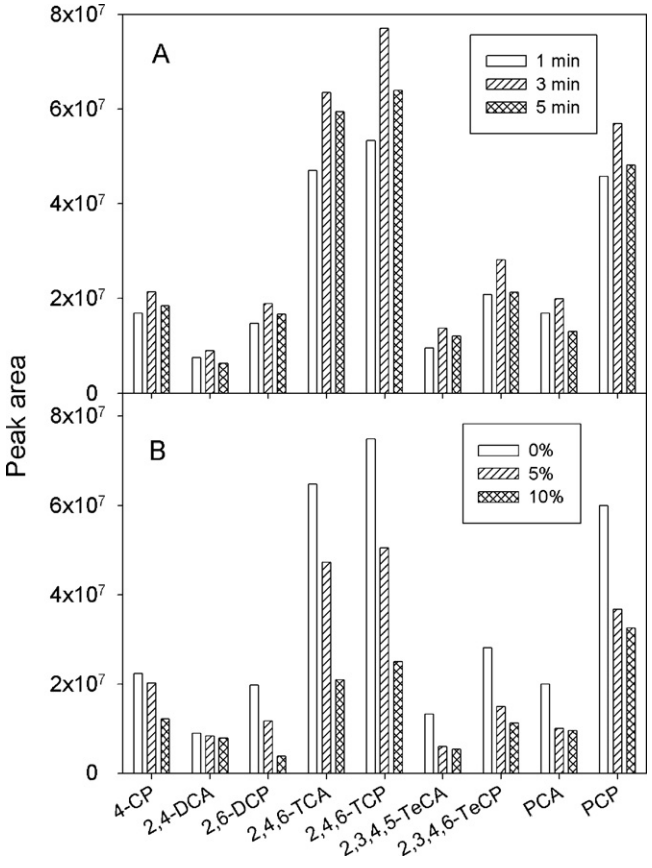

Fig. 2. Influence of (A) centrifugation time and (B) salt concentration on the sensitivity obtained by DLLME. Extraction conditions: aqueous phase, 5 mL; concentration of each compound, 50 ng mL^{-1}.

six different levels) against peak area and following linear regression analysis. Table 2 shows the data obtained. The presence of a matrix effect was confirmed because "*p*" values obtained from application of a paired *t*-test were lower than 0.05 for all the analytes. The detection limits obtained by aqueous calibration by means of DLLME-GC–MS for haloanisole compounds pointed to an increase in sensitivity, of between 380- and 700-fold (for 4-CA and 2,6-DCA, respectively), compared with that obtained in the absence of a preconcentration step. This comparison was not made for chlorophenols because the derivatization step in aqueous medium involves preconcentration. A synthetic wine was used for calibration purposes, and the results (Table 2) show that the matrix effect was not overcome ("*p*" values ranging from 0.002 to 0.049 in most cases). The compound 2,4-dibromoanisole was assayed as internal standard, after checking that all samples were free of the same. When the internal standard was added at 50 ng mL^{-1} significant differences between the slopes of the graphs still remained for both haloanisoles and chlorophenols. Nevertheless the correlation coefficients for all the calibration graphs improved significantly for all the analytes, and so this compound was adopted to improve reproducibility.

Table 3
Analytical characteristics of the method for wine samples.

Compound	Detection limit, ng mL^{-1}		RSD, %	
	Red wine 1	White wine 1	Red wine 1	White wine 1
4-CA	0.011	0.010	2.5 (10)	5.2 (10)
2,6-DCA	0.010	0.011	7.5 (10)	8.1 (10)
4-CP	0.005	0.004	10.1(10)	10.5 (10)
2,4-DCA	0.010	0.009	9.6 (10)	9.9 (10)
2,4,6-TCA	0.009	0.007	4.4 (10)	8.6 (10)
2,6-DCP	0.011	0.010	9.8 (10)	10.4 (10)
2,4,6-TCP	0.024	0.021	5.9 (20)	5.8 (20)
2,4,6-TBA	0.059	0.051	7.8 (20)	10.9 (20)
2,3,4,6-TeCP	0.063	0.041	9.8 (20)	4.3 (20)
2,3,4,5-TeCA	0.095	0.108	10.3 (50)	11.2 (50)
PCA	0.085	0.098	11.4 (50)	9.3 (50)
PCP	0.045	0.041	8.9 (20)	13.2 (20)

Values in parentheses correspond to concentration level, ng mL^{-1}.

The analytical characteristics of the proposed procedure were calculated for two different matrices (red wine sample 1 and white wine sample 1) and the data obtained appear in Table 3. The correlation coefficients (r^2) were in all cases higher than 0.995. Detection limits were calculated from a signal-to-noise ratio of 3. The quantification limits (calculated from a signal-to-noise ratio of 10) varied between 15 pg mL^{-1} for 4-CP and 0.36 ng mL^{-1} for 2,3,4,5-TeCA in

Table 2
Slopes of standard additions calibration graphs, mL ng^{-1}.

Compound	Aqueous	Synthetic wine	Red wine 1	Red wine 2	Red wine 3	White wine 1	White wine 2	White wine 3
4-CA	186,314 (1.631)	62,924 (0.895)	27,062 (3.076)	571,927 (2.271)	74,141 (1.958)	28,550 (2.770)	100,159 (1.759)	256,083 (1.469)
2,6-DCA	155,786 (1.372)	92,806 (1.319)	30,007 (2.937)	655,732 (2.534)	88,113 (2.403)	34,072 (3.282)	169,892 (2.837)	278,786 (1.581)
4-CP	116,753 (1.027)	8807 (0.46)	165,870 (4.533)	168,565 (3.667)	68,421 (1.549)	70,352 (1.678)	13,186 (1.216)	35,977 (1.194)
2,4-DCA	201,939 (1.76)	109,149 (1.563)	32,788 (2.776)	717,565 (2.891)	119,902 (4.135)	32,333 (3.136)	131,381 (2.213)	506,082 (2.347)
2,4,6-TCA	133,247 (1.166)	33,939 (0.484)	12,847 (2.511)	10,857 (1.189)	8394 (0.196)	4256 (0.411)	6204 (0.104)	14,717 (1.183)
2,6-DCP	73,826 (0.648)	3889 (0.052)	14,183 (2.232)	169,453 (0.677)	194,089 (5.333)	7174 (1.083)	445,384 (7.565)	713,330 (4.137)
2,4,6-TCP	297,862 (2.609)	87,070 (1.246)	72,923 (3.095)	858,522 (3.470)	79,693 (2.327)	44,328 (4.303)	181,787 (3.067)	161,573 (0.910)
2,4,6-TBA	101,375 (0.887)	16,842 (0.237)	2525 (0.606)	125,180 (0.488)	28,108 (0.670)	7630 (0.375)	41,940 (0.706)	98,375 (0.555)
2,3,4,6-TeCP	103,489 (0.909)	39,635 (0.564)	28,725 (2.391)	457,059 (1.786)	95,514 (2.739)	17,356 (1.675)	199,609 (3.539)	673,645 (3.805)
2,3,4,5-TeCA	137,516 (1.22)	33,946 (0.484)	6409 (0.523)	130,242 (0.483)	20,137 (0.453)	7753 (0.505)	33,744 (0.560)	73,427 (0.416)
PCA	144,603 (1.272)	20,354 (0.284)	3117 (0.667)	90,743 (0.627)	4597 (0.108)	4001 (0.235)	8737 (0.146)	69,305 (0.397)
PCP	31,472 (0.185)	62,063 (0.814)	37,012 (3.067)	710,361 (3.067)	62,352 (1.462)	24,117 (1.432)	192,335 (3.175)	273,182 (1.529)

Values in parentheses correspond to slopes using the internal standard method.

Table 4
Analytical characteristics of the internal standard method for cork stoppers.

Compound	Slope	Correlation coefficient	Detection limit, pg g^{-1}	Quantification limit, ng g^{-1}	RSD, %
4-CA	0.947 ± 0.020	0.9998	80	0.26	5.4 (5)
2,6-DCA	0.853 ± 0.018	0.9993	86	0.29	4.2 (5)
4-CP	0.979 ± 0.012	0.9998	92	0.31	5.9 (5)
2,4-DCA	4.122 ± 0.194	0.9967	71	0.24	6.9 (5)
2,4,6-TCA	0.361 ± 0.013	0.9979	66	0.22	6.6 (5)
2,6-DCP	3.744 ± 0.065	0.9995	63	0.21	3.3 (5)
2,4,6-TCP	7.649 ± 0.155	0.9994	24	0.08	5.3 (3)
2,4,6-TBA	0.146 ± 0.003	0.9993	32	0.11	6.1 (3)
2,3,4,6-TeCP	5.820 ± 0.069	0.9998	40	0.13	6.2 (3)
2,3,4,5-TeCA	0.250 ± 0.007	0.9987	100	0.33	7.8 (5)
PCA	0.664 ± 0.060	0.9996	220	0.73	4.9 (10)
PCP	3.222 ± 0.125	0.9977	110	0.37	3.4 (5)

Values in parentheses correspond to concentration level, ng g^{-1}.

white wine. The repeatability was calculated by using the relative standard deviation from a series of 10 DLLME-GC–MS consecutive analyses of a red and a white wine under the optimized conditions for each type of matrix and fortified at the concentration levels specified in Table 3.

3.4.2. Cork samples

For quantification purposes calibration was carried out by preparing aqueous standards at six concentration levels in the presence of 50 ng mL^{-1} of 2,4-DBA (IS), submitting 5 mL of each standard solution to the DLLME-GC–MS optimized procedure but using 2 mL of the disperser solvent containing 30 μL of CCl_4. Table 4 shows the analytical characteristics of the adopted method which provided correlation coefficients higher than 0.9967 for all the analytes. The detection and quantification limits were calculated using the same criteria adopted for wine samples. The repeatability of the proposed method was demonstrated by repetitive analyses, calculating the average relative standard deviation for 10 successive analysis of 2.5 g aliquots of ground cork free of the analytes that were previously fortified and submitted to the extraction and DLLME steps. The results obtained were used to evaluate the precision of the method and are shown in Table 4.

3.5. Real samples and validation of the methods

The optimized procedures were applied to a total of 5 red wines, 5 white wines and their corresponding 10 cork stoppers. The results showed that the commercial analyzed wines and also the corks were free of the compounds under study, or at least above the corresponding detection limits. On the other hand, the red wine sample in which taint defect had been detected by sensory analysis was analyzed by the DLLME-GC–MS procedure proposed, and provided analytical signals for seven of the studied compounds: 1.8 ± 0.1 ng mL^{-1} for 4-CA, 1.9 ± 0.1 ng mL^{-1} for 2,6-DCA, 1.7 ± 0.1 ng mL^{-1} for 4-CP, 1.9 ± 0.1 ng mL^{-1} for 2,4-DCA, 2.2 ± 0.2 ng mL^{-1} for 2,4,6-TCA, 8.8 ± 0.3 ng mL^{-1} for 2,4,6-TCP and 2.7 ± 0.2 ng mL^{-1} for 2,3,4,6-TeCP.

To test the applicability and accuracy of the proposed method in real samples analysis, four different wines (two red wines and two white wines) and 10 samples of unused cork stoppers samples were fortified at two concentration levels (corresponding to 5- and 10-fold the corresponding quantification limits) and the corresponding extraction (in the case of cork samples) and DLLME optimized method were carried out. The results showed recoveries of 98.1 ± 12.8% (n = 192) for wine matrices and 104.9 ± 15.2% (n = 72) for the corks (Table 5).

Table 5
Recovery percentages of the analytes from wines and corks.

Compound	Concentration level, ng mL^{-1}	Red wine[a] 1	Red wine[a] 2	White wine[a] 1	White wine[a] 2	Cork[b]
4-CA	0.05	81.4	82.1	96.2	111	82.1
	0.10	82.0	86.3	103	101	114
2,6-DCA	0.05	81.3	119	90	88.3	86.3
	0.10	90.5	117	102	94.5	119
4-CP	0.02	110	117	103	81.1	91.4
	0.05	96.9	89.5	93.2	89.6	117
2,4-DCA	0.05	89.3	97.1	91.9	109	116
	0.10	91.2	106	99.2	95.8	102
2,4,6-TCA	0.05	109	118	91.6	118	118
	0.10	83.4	97.2	101	89.0	91.2
2,6-DCP	0.05	86.3	112	81.0	82.6	83.4
	0.10	91.4	119	87.4	89.2	112
2,4,6-TCP	0.10	103	109	118	118	115
	0.20	96.5	99.3	89.2	82.1	96.8
2,4,6-TBA	0.30	88.1	106	80.9	81.7	113
	0.60	81.9	103	101	92.6	96.5
2,3,4,6-TeCP	0.30	119	82.1	114	118	83.6
	0.60	116	91.4	119	111	88.1
2,3,4,5-TeCA	0.5	112	83.6	118	84.3	116
	1.0	85.3	81.4	94.4	89.3	119
PCA	0.5	89.3	117	85.3	88.0	117
	1.0	98.6	99.8	112	114	115
PCP	0.25	118	82.3	92.1	119	94.3
	0.5	86.1	101	107	116	109

[a] n = 2.
[b] n = 3.

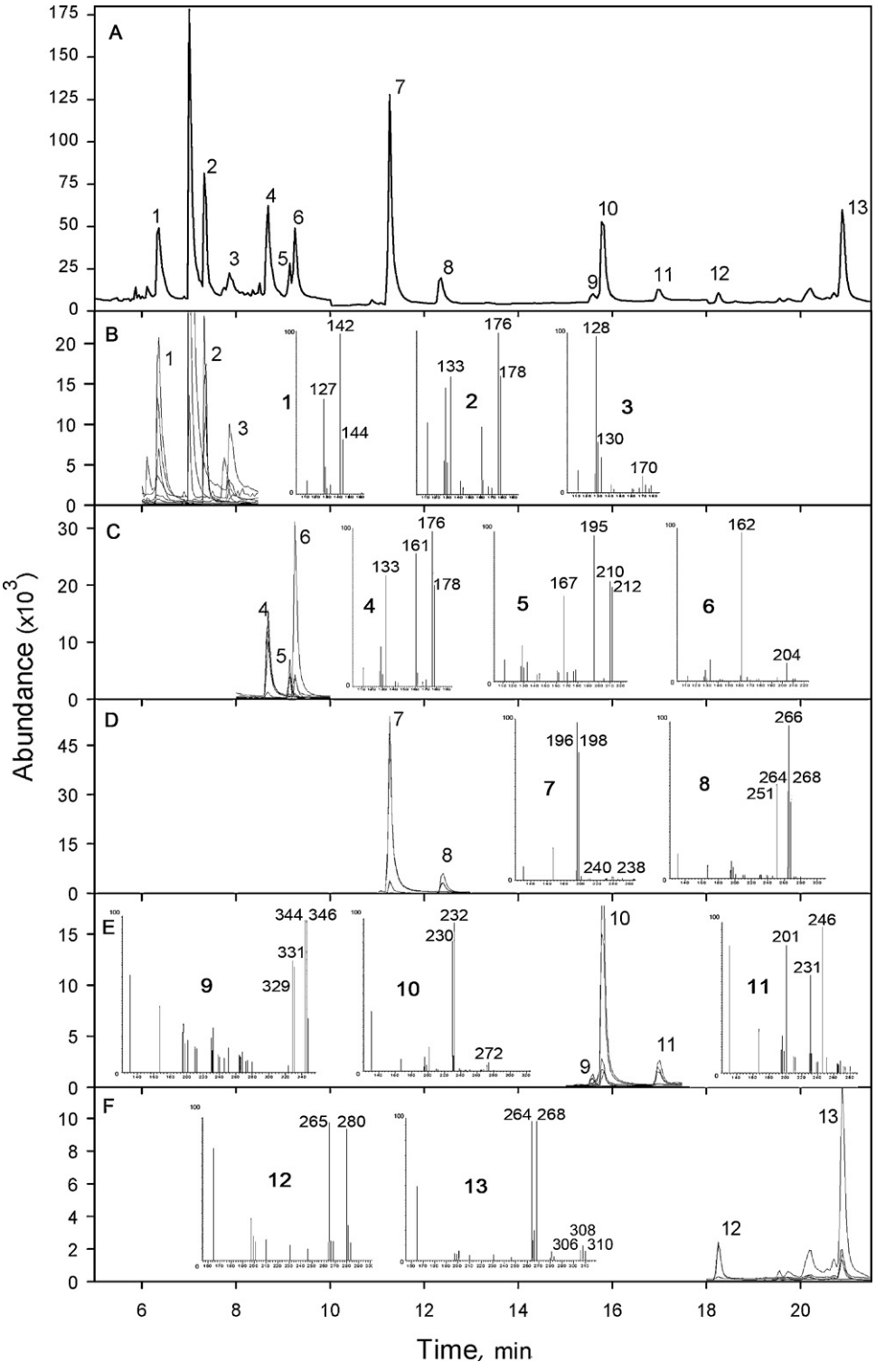

Fig. 3. (A) Elution profile obtained for a spiked red wine sample by DLLME-GC–MS under SIM mode. Peaks correspond to: (1) 4-CA; (2) 2,6-DCA; (3) 4-CP; (4) 2,4-DCA; (5) 2,4,6-TCA; (6) 2,6-DCP; (7) 2,4,6-TCP; (8) 2,4-DBA; (9) 2,4,6-TBA; (10) 2,3,4,6-TeCP; (11) 2,3,4,5-TeCA; (12) PCA; and (13) PCP. (B–F) Extracted ion chromatograms showing the spectra of compounds.

Fig. 3A shows a typical chromatographic profile obtained using DLLME-GC–MS in SIM mode for a fortified red wine sample in the selected conditions. Similar chromatograms were obtained for the other wine samples. Fig. 3B–F shows the corresponding extracted chromatograms.

The extraction percentage of the studied analytes from the cork stoppers into the wine samples was estimated by analyzing the cork macerate obtained as described in Section 2. Aliquots of 2.5 mL of the macerate solution were submitted to acetylation and the same DLLME procedure adopted for red wines applied. Calibration was carried out against aqueous standards prepared in a cork macerate medium obtained with unfortified ground cork samples, which were previously confirmed to be free from the analytes. The transference percentage obtained for each analyte under the specified

Table 6
Results obtained from the analysis of macerate solutions.

Compound	Concentration added, $\mu g\,g^{-1}$	Concentration found[a], $ng\,mL^{-1}$	Mean extraction percentage
2,6-DCA	0.2	4.36 ± 0.23	21.8
4-CP	0.2	2.06 ± 0.17	10.3
2,4-DCA	0.2	1.61 ± 0.14	8.0
2,4,6-TCA	0.2	3.03 ± 0.24	15.2
2,6-DCP	0.2	3.31 ± 0.31	16.6
2,4,6-TCP	0.2	5.36 ± 0.29	26.8
2,4,6-TBA	0.2	2.61 ± 0.15	13.1
2,3,4,6-TeCP	0.2	3.31 ± 0.16	16.5

[a] Mean value ± standard deviation ($n = 3$).

conditions appears in Table 6, and was in all cases lower than 27%. The compounds that were not detected in the macerate solution do not appear in Table 6.

3.6. Comparison of DLLME with other sample preparation techniques

The developed microextraction technique has distinct advantages over conventional methods, already applied to the purpose here presented, such as LLE [3,10] and SPE [4,23,38,42], concerning specially to extraction time and volume of both sample and organic solvents. This proposed sample preparation is much simpler than the conventional approaches and also cheaper than other solvent-free methods, such as SPME [5–9,11,12,16,18,22,24–26,36,37] and SBSE [13,17] which require larger extraction times. On the other hand, detection limits in the order of 10 times higher than those attained with LLE, SPE, SPME and SBSE are obtained for most compounds with DLLME, although this sensitivity is enough in order for the procedure to be applied for routine monitoring of wines and cork samples.

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